High-Throughput Analysis of an Immunosuppressant Drug Panel in Whole Blood Using Ultrafast SPE/MS/MS.

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Abstract

Introduction: In many clinical research laboratories, liquid chromatography-mass spectrometry (LC/MS) methods of analysis of immunosuppressant drugs have proven superior because of their increased sensitivity and selectivity. We evaluated the ability of an ultrafast SPE/MS/MS system to simultaneously analyze tacrolimus, everolimus, sirolimus, and cyclosporin A in whole blood with much faster sample cycle times and similar analytical results compared to LC/MS/MS assays.
Methods: MS methods for tacrolimus, everolimus, sirolimus, and cyclosporin A and their corresponding internal standards were optimized for analysis by QQQ MS. Calibration standards for each analyte were prepared in bovine whole blood. The whole blood samples were mixed with water and precipitated using a zinc sulfate and methanol solution containing the internal standards. Precipitated samples were gently mixed and then centrifuged. Following centrifugation, supernatants were transferred to a 96-well plate for analysis. Samples were analyzed using an Agilent RapidFire high-throughput mass spectrometry system coupled to a QQQ mass spectrometer. A C18 column was used for online SPE.
Results: Prepared calibration standards were run in triplicate over a series of days to establish both intra- and inter-day precision and accuracy. Cyclosporin A, for example, had both intra- and inter-day accuracies within 15% and coefficient of variation values less than 6% for all concentrations within the linear range (7.8-1000 ng/ml). The method for all four analytes had excellent linearity within their respective measured ranges with an R^2 value greater than 0.995. Signal-to-noise ratios were calculated by looking at peak to peak height and found to be greater than 40:1 at the limit of quantitation for all four analytes. To further evaluate this method, identical human samples were analyzed by RapidFire and a traditional LC/MS/MS method. Excellent correlation was found for the two methods.
Conclusions: Based on these results: tacrolimus, everolimus, sirolimus, and cyclosporin A can be accurately and precisely measured in whole blood. All four immunosuppressant drugs were simultaneously analyzed in a 12 MRM panel in less than 13 seconds per sample using ultrafast SPE/MS/MS. While the analytical results were comparable to LC/MS/MS, the analysis time was approximately 10 times faster. This methodology is capable of throughputs >270 samples per hour.

Introduction and Experimental

We evaluated the ability of an ultra-fast SPE/MS/MS system to analyze tacrolimus, everolimus, sirolimus, and cyclosporin A in whole blood. The SPE/MS/MS system consisted of an Agilent RapidFire 360 and an Agilent 6460 triple quadrupole mass spectrometer. The RapidFire cycle consisted of five states: aspirate, load/aqueous wash, extra wash with 50% methanol, elute, and re-equilibration (Table 1). All four immunosuppressant drugs and their corresponding internal standards were simultaneously measured using a 12 MRM panel (Table 2).

Table 1. RapidFire Conditions

RapidFire Conditions
Buffer A: 10 mM ammonium acetate + 0.09% Formic acid + 0.01% TFA
Buffer B: 50% Methanol
Buffer C: 10 mM ammonium acetate in methanol + 0.09% FA + 0.01% TFA
10µl injection
C18 Agilent RapidFire SPE cartridge
RF State 1 (aspirate): sip sensor
RF State 2 (load/wash) 3000 ms
RF State 3 (extra wash) 2000 ms
RF State 4 (elute) 3500 ms
RF State 5 (re-equilibrate) 500 ms

Table 2. 12 MRM Panel

Analyte	Q1	Q3	Dwell	Fragmentor	CE
Tacrolimus quantifier	821.9	768.5	10	145	17
Tacrolimus qualifier	821.9	786.5	10	145	13
Ascomycin	809.6	756.5	10	125	17
Everolimus quantifier	975.6	908.6	10	170	15
Everolimus qualifier	975.6	926.8	10	170	10
Everolimus-d4	979.6	912.7	10	180	10
Sirolimus quantifier	931.6	864.6	10	175	12
Sirolimus qualifier	931.6	882.2	10	175	8
Sirolimus-d3	934.6	864.5	10	180	18
Cyclosporin A quantifier	1219.8	1202.8	10	170	12
Cyclosporin A qualifier	1202.9	1184.8	10	200	30
Cyclosporin A-d4	1223.9	1206.9	10	170	12

Experimental

Calibration standards for tacrolimus, sirolimus, everolimus (0.8-50 ng/mL), and cyclosporin A (15.6-1000 ng/mL) were prepared by spiking all four drugs into bovine whole blood. Commercially available quality control standards made in whole blood were prepared according to the manufacturer's instructions. The whole blood samples were mixed with water and precipitated using a zinc sulfate and methanol solution containing the internal standards. Precipitated samples were gently mixed and then centrifuged. Following centrifugation, supernatants were transferred to a 96-well plate for analysis.

Table 3. QQQ MS Conditions

QQQ Conditions			
Gas temperature	225°C	Sheath gas temperature	325°C
Gas flow	9 L/min	Sheath gas flow L/min	12
Nebulizer	40 psi	Nozzle voltage	300 V
Sheath gas temperature	325°C	Capillary voltage	4000 V

Results and Discussion

	Intraday (n=3)		Interday (n=4)	
ng/ml	% Precision	% Accuracy	% Precision	% Accuracy
15.6	3.6	95.9	2.4	97.5
31.3	0.4	94.2	0.7	93.4
62.5	2.2	95.3	1.2	95.7
125	0.5	98.9	4.5	101.2
250	4.9	99.8	1.3	98.9
500	4.4	104.3	1.7	102.3
1000	2.2	98.5	1.5	99.4
Low QC	1.0	101.0	3.8	98.3
Mid QC	5.0	94.0	3.3	96.4
High QC	2.3	97.6	3.7	98.0

Figure 1. Representative standard curve and Intra- Interday data for Cyclosporin A

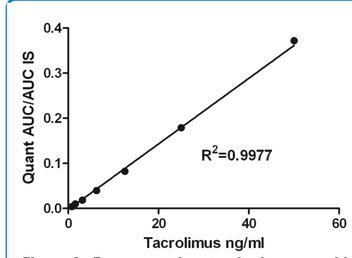
	Intraday (n=3)		Interday (n=4)	
ng/ml	% Precision	% Accuracy	% Precision	% Accuracy
0.8	6.5	101.4	3.0	104.4
1.6	9.6	97.2	4.9	94.1
3.1	3.7	90.3	1.2	89.6
6.3	6.9	93.0	1.6	92.1
12.5	6.2	87.9	3.0	91.6
25	0.7	99.6	0.9	99.1
50	1.5	104.6	0.5	104.1
Low QC	8.1	103.2	4.2	98.4
Mid QC	4.3	95.7	2.8	93.5
High QC	9.7	94.3	4.5	95.7

Figure 2. Representative standard curve and Intra- Interday data for Everolimus

	Intraday (n=3)		Interday (n=4)	
ng/ml	% Precision	% Accuracy	% Precision	% Accuracy
0.8	2.8	109.0	2.6	106.2
1.6	4.4	94.0	4.9	96.1
3.1	8.3	92.2	6.4	85.3
6.3	6.2	86.9	6.0	91.9
12.5	9.6	90.6	3.3	91.6
25	5.6	96.2	2.2	95.4
50	1.9	106.2	0.6	106.2
Low QC	7.0	91.3	3.4	95.9
Mid QC	7.6	101.9	1.9	101.7
High QC	7.3	100.1	1.8	98.6

Figure 3. Representative standard curve and Intra- Interday data for Sirolimus

Results and Discussion

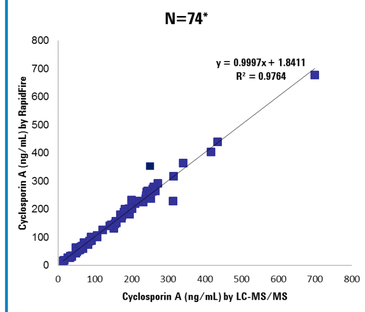


	Intraday (n=3)		Interday (n=4)	
ng/ml	% Precision	% Accuracy	% Precision	% Accuracy
0.8	7.0	97.4	8.1	106.7
1.6	5.2	97.3	4.6	91.5
3.1	1.9	90.1	3.4	88.9
6.3	1.1	91.9	3.0	88.8
12.5	1.3	91.8	2.6	92.2
25	1.4	98.3	1.9	98.0
50	0.9	104.4	1.0	105.0
Low QC	0.7	99.1	1.4	99.4
Mid QC	4.3	97.2	1.8	96.7
High QC	3.1	97.6	1.9	96.3

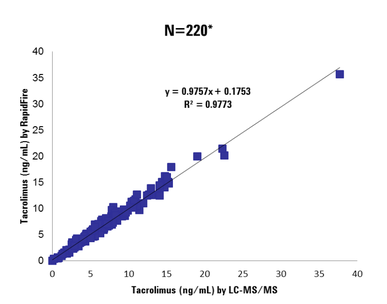
Figure 4. Representative standard curve and Intra- Interday data for Tacrolimus

The RapidFire method for all four analytes had excellent linearity within their respective measured ranges with an R^2 value greater than 0.995. Signal-to-noise ratios were calculated by looking at peak to peak height and found to be greater than 40:1 at the limit of quantitation for all four analytes.
To further evaluate this method, identical human samples were analyzed. The human samples were independently analyzed by LC/MS/MS at UCL and aliquots of identical samples analyzed by RapidFire. Excellent correlation was found for the two methods.

Figure 5. Correlation curves of human samples analyzed by LC/MS/MS vs. RapidFire SPE/MS/MS



*1 outlier removed due to potential stability issues during shipment



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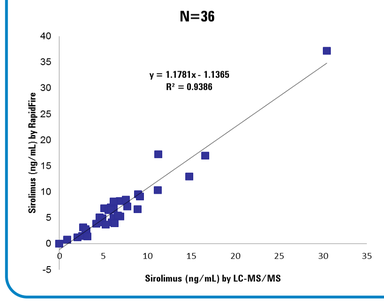

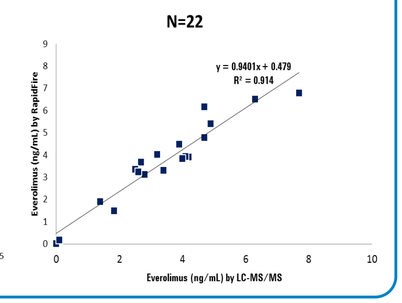

Conclusions

Cyclosporin A, everolimus, sirolimus, and tacrolimus were accurately and precisely measured in whole blood. All four immunosuppressant drugs were simultaneously analyzed in a 12 MRM panel in less than 13 seconds per sample. Human samples correlated well with identical samples ran independently by a traditional LC/MS/MS method. While the analytical results were comparable to LC/MS/MS, the analysis time was approximately 10 times faster. This methodology is capable of throughputs >270 samples per hour.

Note: The method described in this presentation is for research purposes only and not approved for diagnostic use